Original article

Antimonial treatment of visceral leishmaniasis: are current in vitro susceptibility assays adequate for prognosis of in vivo therapy outcome?

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Abstract

In most of the Indian subcontinent, the first line treatment for visceral leishmaniasis (VL) is sodium stibogluconate (SSG), an antimonial drug, but the efficacy of the drug varies according to region. We aimed to characterize the in vitro antimony susceptibility of clinical isolates of Nepalese VL patients, and to correlate this in vitro parasite phenotype to clinical therapy outcome. Thirty-three clinical isolates of L. donovani were taken from patients with known disease history. These isolates were typed and the susceptibility of intracellular amastigotes to pentavalent (SbV) and trivalent (SbIII) antimonials was determined. We observed (i) 22 SbV-resistant isolates out of 33 tested and (ii) 3 SbIII-resistant isolates out of 12 tested. Amongst the latter, there were three combinations of in vitro phenotypes: (i) parasites sensitive (n = 4) or (ii) resistant to both drugs (n = 3) and (iii) resistant to SbV only (n = 5). There was no geographical clustering in terms of in vitro susceptibility. The relation between the in vitro susceptibility to antimonials and the corresponding in vivo treatment outcome was ambiguous. Our results highlight the need to adjust the currently used Leishmania drug susceptibility assays if they are to be used for prognosis of in vivo SSG treatment outcome.

Keywords: Leishmania donovani; Pentavalent and trivalent antimonials; Kala-azar; Nepal; Drug resistance

1. Introduction

Visceral leishmaniasis (VL) or kala-azar affects an estimated 500 000 persons every year worldwide, with 50% of the cases reported from Bangladesh, India and Nepal [1] and 100% fatality when untreated. The first line treatment for VL is a pentavalent antimonial (sodium stibogluconate or meglumine antimonate) as recommended by the World Health Organization [2]. In the Indian sub-continent, the efficacy of sodium stibogluconate (SSG) has gradually declined in spite of regularly increasing both doses and duration of treatment. However, there is a geographical variability in efficacy even within this region. In India for instance, the SSG cure rate was 86% in the state Uttar Pradesh but only 35% in some districts of Bihar (a state eastern to Uttar Pradesh) in the period 1994—1997 [3]. At that time, it was recommended to replace SSG by amphotericin B as first line treatment in the SSG refractory districts of Bihar [4]. Similarly, in South East Nepal, the average efficacy of SSG treatment was reported to be 90% between 1999 and 2000, but specifically in Nepalese districts bordering Bihar, this value dropped to 76% [5].

Treatment failure may have multiple origins, either related to the drug, the host and/or the parasite itself [6]. However, in...
anthroponotic VL, the emergence of SSG resistant parasites is particularly favored by two conditions. First, the etiological agent *Leishmania donovani* is in frequent contact with the drug, as humans are the main reservoir (anthroponosis). Second, anthropotonic VL is embedded in regions with a poor socio-economic status; as a consequence, treatment may often be sub-optimal, linked to variable drug quality and compliance/accessibility problems [7]. The first antimony-resistant *L. donovani* isolates have indeed been identified in patients from Bihar. Using *in vitro* SSG susceptibility assays, it was shown that isolates from SSG unresponsive patients were three times less sensitive to the drug compared to isolates from SSG cured patients [8]. In eastern Sudan—also endemic for anthroponotic VL—SSG resistant isolates were identified as well; but in contrast to the study done in Bihar, only 27% of isolates from Sudanese relapse patients had a decreased *in vitro* SSG sensitivity compared to the used reference SSG sensitive strain [9]. The apparent discordance between these two studies from India and Sudan could be due to the difference in clinical background of the isolates: immediate unresponsiveness and relapse in India and Sudan, respectively. However, it cannot be excluded that variation in the respective clinical and experimental protocols contributes to differential interpretations.

The current report frames within a multi-disciplinary study (see [http://www.leishmadrug.org](http://www.leishmadrug.org)) which aimed to assess the role of the parasite in SSG treatment failure of anthroponotic VL in Nepal. The VL endemic region in Nepal comprises 12 districts in the southern Terai region with an estimated 6 million people at risk to acquire the disease [10]. The socio-cultural similarity and the open border with northern Bihar facilitate cross border population movements which could play an important role in the spreading of the SSG resistant strains found in Bihar. We obtained 33 clinical isolates of *L. donovani* from 31 Nepalese patients with VL, treated with the generic SSG, sodium antimony gluconate (SGS, Albert David) and presenting different treatment outcomes. We first tested the susceptibility of intracellular amastigotes to pentavalent antimonials (SbV), but also to trivalent antimonials (SbIII), the reduced form of the drug directly toxic to parasites [6]. Second, we compared these *in vitro* data to the treatment outcome of patients from which parasites were isolated.

### 2. Materials and methods

#### 2.1. Patients and clinical protocol

Informed consent was obtained from the patients and in the case of children, from the parents or guardians. Ethical clearance was obtained from the institutional review boards of the Nepal Health Research Council, Kathmandu, Nepal, and the Institute of Tropical Medicine, Antwerp, Belgium. Suspected VL cases (with fever for 2 weeks or longer and splenomegaly) were consecutively recruited in the study from November 2002 to the beginning of 2004 at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal. This institute is a referral centre for the eastern region in Nepal. Individuals less than 2 years old were excluded from the study. Diagnosis of VL was confirmed by demonstration of amastigotes in bone marrow and/or spleen aspirates. All VL patients were treated with SAG (Albert David Ltd, Kolkata), 20 mg/kg per day i.m. ×30 days. All batches of SAG used were tested for quality control by the International Dispensary Association, Amsterdam. Patients received a full supervised course of treatment in the BPKIHS hospital or the nearest public health facility. All patients were followed up for clinical and parasitological evaluation at the end of the 1 month drug course, as well as at 3, 6 and 12 months after start of treatment. Unresponsive patients were given amphotericin B deoxycholate 1 mg/kg per day for 15 days as rescue treatment. Initial cure was defined as a kala-azar case with absence of fever, decreased spleen size and negative parasitology at the end of a 30 days SAG drug course. Non-responders were cases with positive parasitology after a full 30 days SAG drug course. Definite cure was defined as a patient with initial cure who showed no signs and symptoms of relapse at 12 months follow up visit. Relapse was defined as somebody with initial cure but reappearance of clinical signs and positive parasitology during follow up. Treatment failure was defined as either non-response or relapse.

#### 2.2. Parasites and *in vitro* culture

For all patients, parasite isolation was attempted before the start of drug treatment, with an average success rate of isolation of 47.5%. In non-responder or relapsing patients, isolation was also tried post-treatment with an average success rate of 61.1 and 50% respectively. Parasites were typed by PCR-RFLP analysis of cysteine proteinase b [11] and processed as described elsewhere until *in vitro* susceptibility testing within eight passages from isolation [12].

#### 2.3. *In vitro* drug susceptibility testing

Two formulations of SbV were tested: (i) additive-free SSG in powder-form from GSK, UK, which was considered the reference and (ii) SAG from Albert David as was used to treat the patients. Susceptibility testing was done as reported [12], but (i) incubation of infected macrophages occurred at 37 °C and (ii) drugging was done at concentrations of 60, 20, 6.6 and 2.2 μg SbV/ml for a total of 5 days, with the overlay being replaced with fresh dilutions on day 3. The strain *L. donovani* MHOM/ET/67/HU3, a WHO reference strain sensitive to SSG and meglumine antimoniate, was included in each assay as a reference. The activity index (AI) defined previously for comparing results from different experiments (ED50 tested strain/ED50 reference strain [12]) was used. An AI of 1 corresponded to ED50 ranging from 7 to 18 μg SbV/ml, while an AI of 6 corresponded to ED50 higher than 60 μg SbV/ml. Isolates with an AI of 1–2 were considered as sensitive, while those showing an AI of 3 or higher were considered to be resistant. Sensitivity to SbIII was evaluated in the same assay for some of the isolates as described elsewhere [12].

Isolates were selected randomly and tested for their sensitivity to SbV until we reached a minimum of 10 resistant and 10 sensitive isolates. In total, 33 isolates from 31 patients were thus SbV-tested: 26 were pre-treatment isolates and 7 were...
obtained after treatment failure (two patients each providing one pre- and one post-treatment isolate, further called paired isolates). Post treatment was defined broadly: recruited patients (i) without a previous history of treatment with antimonials for whom isolates were available after the directly observed drug therapy of the present study and (ii) with a previous history of SSG treatment and sampled before the directly observed therapy of the present study, labeled with an asterisk in Table 1.

2.4. Data analysis

Demographic, clinical, laboratory, treatment and outcome data were entered in an Excel database. All data were cross-checked with individual patient files during several data monitoring visits by one of the investigators. Statistical analysis was done with SPSS 11.0. for Windows (SPSS Inc., Chicago, IL, USA). When two isolates were available for the same patient (pre- and post-treatment), the in vitro susceptibility result of the pre-treatment isolate was included in the analysis. Categorical variables were compared using cross-tabulations and chi-square or Fisher’s exact test tests, at a critical $\alpha$-level of 0.05. The strength of association of drug-susceptibility testing with clinical outcomes was checked by comparing the risk of treatment failure in patients with susceptible compared to those with resistant parasite strains. Relative risks (RR) with 95% confidence intervals (95% CI) were computed where appropriate.

3. Results

3.1. In vitro susceptibility to SbV and SbIII

The 33 Nepalese isolates analyzed were all identified as L. donovani by cpb PCR-RFLP (not shown). The majority of the isolates came from patients living in the eastern districts of Nepal.

Table 1

Nepalese isolates tested for their in vitro susceptibility to antimonials and link with clinical response

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Origin (district, VDC)</th>
<th>SbV</th>
<th>SbIII</th>
<th>Clinical response</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHOM/NP/02/BPK019/0</td>
<td>Sunsari, Ithari</td>
<td>1</td>
<td>1</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK020/0</td>
<td>Sunsari, Inurwa</td>
<td>1</td>
<td>1</td>
<td>Relapse-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK035/0</td>
<td>Saptari, Bhagani</td>
<td>1</td>
<td>1</td>
<td>Definite cure</td>
<td>Previous complete tx with SAG, cure with ampho B</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK043/0</td>
<td>Sunsari, Barahachhetra</td>
<td>1</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK090/0</td>
<td>Sunsari, Ithari</td>
<td>1</td>
<td>nd</td>
<td>Relapse-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK181/0</td>
<td>Sunsari, Dharapan</td>
<td>1</td>
<td>nd</td>
<td>Relapse-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK080/0</td>
<td>Sunsari, Ithari</td>
<td>1</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK206/0</td>
<td>Sunsari, Ithari</td>
<td>1</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK080/0</td>
<td>Sunsari, Ithari</td>
<td>1</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK294/0</td>
<td>Siraha, Bishnuparkahi</td>
<td>2</td>
<td>0</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK276/0</td>
<td>Sunsari, Ithari</td>
<td>2</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK278/0</td>
<td>Morang, Dharapan</td>
<td>3</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK026/0</td>
<td>Bhojpur, Basteem</td>
<td>3</td>
<td>6</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK191/0</td>
<td>Morang, Sundarpur</td>
<td>4</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK050/0</td>
<td>Sunsari, Chandbela</td>
<td>6</td>
<td>0</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK293/0</td>
<td>Sunsari, Inurwa</td>
<td>6</td>
<td>1</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK087/0</td>
<td>Morang, Bhaudaha</td>
<td>6</td>
<td>1</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK298/0</td>
<td>Sunsari, Ithari</td>
<td>6</td>
<td>1</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK290/0</td>
<td>Siraha, Pipra</td>
<td>6</td>
<td>2</td>
<td>Initial cure</td>
<td>f-up only 1 month (lost afterwards)</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK190/0</td>
<td>Morang, Govindapur</td>
<td>6</td>
<td>6+</td>
<td>Non-responder-</td>
<td>Treated with SAG before admission, no 2nd line tx</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK288/0</td>
<td>Saptari, Sitapur</td>
<td>6+</td>
<td>6+</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK274/0</td>
<td>Sunsari, Ithari</td>
<td>6+</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK275/0</td>
<td>Morang, Sanischare</td>
<td>6+</td>
<td>nd</td>
<td>Non-responder-</td>
<td>Tx failure: non-responder after 25 days SAG</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK279/0</td>
<td>Morang, Sanischare</td>
<td>6+</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK077/0</td>
<td>Saptari, Fatpur</td>
<td>6+</td>
<td>nd</td>
<td>Non-responder-</td>
<td>Previous complete tx with SAG, cure with ampho B</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK085/0</td>
<td>Saptari, Kamalpur</td>
<td>6+</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK164/1</td>
<td>Dhanusa, Dharapan</td>
<td>6+</td>
<td>nd</td>
<td>Non-responder-</td>
<td>Isolate obtained after SAG tx, died during amphoB</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK173/0</td>
<td>Sarlahi, Picari</td>
<td>6</td>
<td>nd</td>
<td>Non-responder-</td>
<td>Tx failure: non-responder after 30 days SAG</td>
</tr>
<tr>
<td>MHOM/NP/02/BPK173/0</td>
<td>Sarlahi, Picari</td>
<td>6</td>
<td>nd</td>
<td>Non-responder-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK177/0</td>
<td>Dhanusa, Govindpur</td>
<td>6</td>
<td>nd</td>
<td>Relapse-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK181/0</td>
<td>Dhanusa, Govindpur</td>
<td>6</td>
<td>nd</td>
<td>Relapse-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK181/0</td>
<td>Dhanusa, Govindpur</td>
<td>6</td>
<td>nd</td>
<td>Relapse-</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK178/0</td>
<td>Sunsari, Inurwa</td>
<td>6+</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
<tr>
<td>MHOM/NP/02/BPK192/0</td>
<td>Morang, Dharapan</td>
<td>6+</td>
<td>nd</td>
<td>Definite cure</td>
<td></td>
</tr>
</tbody>
</table>

Patient had a history of previous treatment with antimonials before being recruited in the present study (relapse-r, relapse after previous treatment; responder-r, non-responder after previous treatment).

a Isolate was not considered for the comparison between SbV-sensitivity and treatment outcome.

b Isolate was not considered for the comparison between SbIII sensitivity and treatment outcome. Tx, treatment; ampho-B, amphotericin B deoxycholate; f-up, follow-up.

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Sunsari and Morang, in the proximity of the BPKIHS reference centre. These two districts border a zone of northern Bihar with mesoendemic VL and with reported SSG cure rates of 64% (Fig. 1) [7,13]. A few isolates originated from Sarlahi and Dhanusa districts, bordering a hyperendemic VL region of Bihar where SSG cure rates drop to 35–48% and SSG resistant L. donovani isolates have been identified (Fig. 1) [8,13].

All the Nepalese isolates were tested for their susceptibility to SbV. The in vitro susceptibility profiles obtained with SAG (the formulation used to treat the patients) were all consistent with the profiles obtained with the reference formulation (additive-free SSG in powder form, GSU). Eleven isolates were classified as SbV-sensitive (SbV-S) and 22 as resistant (SbV-R) (Table 1).

Out of these 33 isolates, 12 (4 SbV-S and 8 SbV-R) could be tested in parallel for their in vitro susceptibility to SbIII, and only 3 isolates were shown to be resistant to the drug (AI of 6 or higher, Table 1). The 9 other isolates showed an AI ranging between 0 and 2 (0, meaning that they were more sensitive to SbIII than the reference strain HU3). When considering the results of SbV and SbIII together, three phenotypes were observed: (i) 4 isolates SbV-sensitive/SbIII-sensitive (further called 5S3S), (ii) 5 isolates SbV-resistant/SbIII-sensitive (5R3S) and (iii) 3 isolates SbV-resistant/SbIII-resistant (5R3R).

We did not observe any micro-geographical clustering in terms of in vitro susceptibility: SbV-resistant isolates were spread out over all districts sampled here. The three 5R3R isolates were found in the districts of Saptari, Morang and Bhojpur at an average distance of 80 km from each other (see Fig. 1). Our collection of isolates allowed us to analyze the distribution of parasites in the micro-focus constituted by the Village Development Committees (VDC) of Ithari, Sundarpur and Dulari (on the border between the districts Sunsari and Morang, Fig. 1), as we obtained isolates from 11 patients from that area; they were living within a 5 km distance from each other. Six isolates were SbV-S and 5 were SbV-R.

3.2. In vitro susceptibility and treatment outcome

In a next stage, we compared the results of the in vitro susceptibility of the clinical isolates of L. donovani to the treatment outcome of the patients from which they were obtained (Table 1).

a. SbV: out of 33 isolates tested in vitro for SbV susceptibility, 4 were excluded from analysis (labeled with ‘a’ in Table 1) because of treatment with amphotericin B (1 patient), unclear clinical outcome (1 patient) and paired isolates (2 patients, in this case, post-treatment isolates were excluded). The 29 remaining isolates belonged to 20 patients with definite cure, and 9 patients with treatment failure (5 non-responders and 4 cases with relapse); the in vitro susceptibility profiles of the corresponding isolates are presented in Fig. 2. All non-responding patients (n = 5) were infected with SbV-R isolates. However, the SbV-R phenotype did not necessarily cause treatment failure as 13/19 SbV-R isolates came from patients with definite cure. Thus, the risk for immediate SSG unresponsiveness when infected with SbV-R parasites is 5/19 (26.3%), while it was 0% (0/10) when infected with SbV-S parasites. Relapse patients on the other hand were found to be infected with SbV-S or SbV-R isolates. The risk of clinical
Clinical profile patients vs SbV in vitro profile isolates:

- 20 definite cure
- 5 non-responder
- 4 relapse

- 7 SbV-S
- 13 SbV-R
- 0 SbV-R
- 5 SbV-R
- 3 SbV-R
- 1 SbV-R

Clinical profile patients vs SbIII in vitro profile isolate:

- 2 treatment failure
- 9 definite cure
- 1 non-responder
- 1 relapse

- 7 SbIII-S
- 2 SbIII-R
- 0 SbIII-R
- 1 SbIII-R
- 1 SbIII-R
- 0 SbIII-R

Fig. 2. Relationship between treatment outcome of respective patients and in vitro SbV/SbIII susceptibility of respective clinical isolates. Dark boxes: patients SSG treatment outcome; light boxes: susceptibility profile of corresponding isolates; isolates labeled with ‘a’ or ‘b’ in Table 1 are not included; numbers in the symbols: number of isolates of the same phenotype observed in a given locality.

4. Discussion

We demonstrated here that SbV-R parasites are present in the VL endemic region in Nepal, as in the neighboring Indian region Bihar. However, it is unclear at this stage to what degree the phenomenon of natural resistance in Nepal is related to the resistance problem in Bihar. We did not find clustering of resistant isolates close to the Bihar border, but our sample size was small and not equally distributed over the different districts. The few isolates studied from the districts Sarlahi to Saptari bordering the highly SSG unresponsive region in Bihar were SbV resistant (Fig. 1), but that sample is probably biased as it is likely to mainly concern referral patients from the local district hospitals (50–200 km travel to BPKHIS). We found no cross-resistance to other drugs, amphotericin B (unpublished observations) and miltefosine [14], as in Bihar [15,16]. The absence of cross-resistance is reflected on the clinical level as SSG unresponsive/relapse cases can be cured with alternative treatments in Nepal (Table 1) and Bihar [7].

We observed a major discrepancy with the previous report based on the analysis of 24 isolates from Bihar [8]. This concerned the association between the SbV-susceptibility and the clinical outcome of therapy: correlation (Indian study) and absence of correlation (this study). A similar contradiction was observed between two recent studies on New World species [14,17]. In both epidemiological settings, discrepancies concerned specifically the link between SbV resistance and treatment failure: correlation [8,17] or absence of correlation (results from our group: this study and [14]). These discrepancies could be explained by several factors, operational and/ or biological.

Study protocols and definitions differed between studies. Lira et al. reported that drugging was done after 3 days of macrophage infection, and determination of ED50 was made 3 days later [8]. In the present study, drugging was done at days 1 and 3 respectively and ED50 measurement at day 5. We also introduced in each experimental set the same reference sensitive strain, in order to take into account inter-experimental variations and normalize the ED50 of clinical isolates to that of the reference; applying this method, we only considered as SbV-resistant the isolates which were more than six times less sensitive than HU3. In Lira’s study, amastigotes from unresponsive strains were an average of three times more resistant than the sensitive ones. If our threshold had been applied by these authors, the correlation with treatment failure would have been weaker. Clinical definitions were also partially different in both studies (definite cure after 6 months follow-up [8] and 12 months in the present study), which could contribute to explaining some differences.

Discrepancies could also be explained by biological differences among Indian and Nepalese SbV-R isolates, which would not be detected by the current in vitro assays. To understand it, the proposed dual action mode of SSG should be repeated: (i) SSG is converted to SbIII, which in turn has direct leishmanicidal activity [18,19], and (ii) SSG stimulates infected cells to produce microbicidal compounds such as ROI and nitric oxide which kill the intracellular parasites [20,21]. On the one hand, SbIII susceptibility was not analyzed in previous studies on _L. (L.) donovani_, but here this was done on a sub-sample of isolates. We demonstrated here the existence of SbIII-S as well as SbIII-R isolates, and most of all that SbV-resistance was not necessarily correlated with SbIII-resistance (57% and 43% of 5R3S and 5R3R isolates respectively). However, our sample size was too small to test if parasites resistant to both components were significantly more associated with treatment failure. We recommend to testing more
Nepalese strains with SbIII and most of all re-test historical strains from Bihar. Indeed, considering that SSG treatment failure is well installed in this region, it is possible that the SbIII-R phenotype is much more abundant than in Nepal and that this constituted a confounding factor in Lira’s report. On the other hand, considering the stimulating effect of SSG and that this constituted a confounding factor in Lira’s report.

In comparison, the in vitro system used for susceptibility assays does not include any immune components. It is possible that Indian and Nepalese in vitro SbV resistant parasites have a different molecular mechanism of SbV resistance, one mechanism which only confers resistance in vitro (as currently measured), and yet another mechanism which confers resistance in vitro and in vivo. Characterization of underlying molecular mechanisms of resistance is required to resolve this issue.

The relation between the identified in vitro phenotypes and the in vivo treatment outcome is not always straightforward and thereby questions the adequacy of the current Leishmania in vitro susceptibility assays for prognosis of treatment outcome in vivo. In vitro assays should be adjusted by (i) systematically including SbIII testing and (ii) including some cytokines to mimic the synergistic effect of the immune system in vivo. However, specific molecular characterization of the different antimonal resistant phenotypes could be even more favorable and would contribute to a more accurate prognosis of a parasite’s in vivo behavior and response to SSG treatment.

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